

**Methanotrophs and Methanotrophic Activity in Engineered Landfill
Biocovers**

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Abstract

The dynamics and changes in the potential activity and community structure of methanotrophs in landfill covers, as a function of time and depth were investigated. A Passive Methane Oxidation Biocover (PMOB-1) was constructed in St-Nicéphore MSW Landfill (Quebec, Canada). The most probable number (MPN) method was used for methanotroph counts, methanotrophic diversity was assessed using denaturing gradient gel electrophoresis (DGGE) fingerprinting of the *pmoA* gene and the potential CH₄ oxidation rate was determined using soil microcosms. Results of the PMOB-1 were compared with those obtained for the existing landfill cover (silty clay) or a reference soil (RS). During the monitoring period, changes in the number of methanotrophic bacteria in the PMOB-1 exhibited different developmental phases and significant variations with depth. In comparison, no observable changes over time occurred in the number of methanotrophs in the RS. The maximum counts measured in the uppermost layer was 1.5×10^9 cells g dw⁻¹ for the PMOB-1 and 1.6×10^8 cells g dw⁻¹ for the RS. No distinct difference was observed in the methanotroph diversity in the PMOB-1 or RS. As expected, the potential methane oxidation rate was higher in the PMOB-1 than in the RS. The maximum potential rates were 441.1 and 76.0 µg CH₄ h⁻¹g dw⁻¹ in the PMOB and RS, respectively. From these results, the PMOB was found to be a good technology to enhance methane oxidation, as its performance was clearly better than the starting soil that was present in the landfill site.

Key words: Methane oxidation, Landfill Cover Soil, *pmoA*, DGGE, Phylogenetic Tree, Potential Methanotroph Activity, Methanotrophs

1. Introduction

Methane (CH_4) is one of the most important greenhouse gases, with a global warming potential 25 times higher than that of CO_2 (averaged over 100 years) (IPCC 2007). In landfills, anaerobic biodegradation of solid wastes by methanogens is responsible for about 19% of the anthropogenic CH_4 introduced into the atmosphere each year, contributing to approximately 40 ± 20 Tg of the global CH_4 emissions (Lelieveld et al. 1998). This source of methane emissions partly results from the lack of a gas collection system in thousands of old landfills and uncontrolled dumps around the world.

Among design advances to reduce CH_4 emissions, gas collection systems have greatly reduced the environmental impact of new landfills and are now mandatory in most parts of the world. However, installation of a gas collection system in small or old landfills with low CH_4 production is not economically feasible (Mor et al. 2006; Streese and Stegmann 2003), and in new landfills, gas collection systems are not 100% efficient (Spokas et al. 2006). This means that there will always be a certain amount of fugitive emissions. Therefore, any technology or approach that could help reduce atmospheric emissions of CH_4 from old or new landfills will make an important contribution to reducing the overall atmospheric CH_4 budget.

Proper use of techniques pertaining to the fields of geoenvironmental engineering and biotechnology can optimize the methane oxidation process within the landfill cover soil. More precisely, covering landfills using materials that might offer promising conditions for the development of methane oxidizing bacteria or methanotrophs would be the equivalent of installing an immense biofilter above the waste mass, here referred to as a passive methane oxidation biocover (PMOB). PMOB efficiency depends on soil conditions including the type of soil and porosity. Substrates must have a suitable pore volume to ensure the satisfactory supply

1 of oxygen and methane as well as an adequate retention time for methane within the substrate
2 (Humer and Lechner 2001). Substrates must also have a minimum of organic matter content and
3 provide a satisfactory supply in nutrients that are essential prerequisites for the growth and
4 function of methanotrophic biomass (Czepiel et al. 1995). Previous studies have shown that
5 microbial CH₄ oxidation in landfill cover soil can be enhanced when using substrates that are
6 rich in organic matter, such as compost, rather than pure clay covers (Abichou et al. 2006; Stern
7 et al. 2007).

8 Natural CH₄ oxidation by methanotrophs, which have been isolated and characterized from a
9 variety of soils, provides an important biological sink for the CH₄ that migrates through the
10 landfill-cover soil (Kightley et al. 1995; Whalen et al. 1990). Methanotrophs are aerobic
11 microorganisms that use oxygen to oxidize CH₄ to CO₂ and biomass (Hanson and Hanson 1996).
12 Methanotrophs oxidize methane to methanol using the enzyme, methane monooxygenase
13 (MMO). There are two distinct forms of MMO: the particulate membrane-bound form (pMMO)
14 and the soluble form, sMMO. The *pmoA* is a functional gene encoding the active site subunit of
15 pMMO for all known methanotrophs (Lieberman and Rosenzweig 2004) with the possible
16 exception of members of the genus *Methylocella*, which are generally isolated from acidic
17 environments (Dunfield et al. 2003; Theisen et al. 2005). The *pmoA* gene has been used as a
18 marker in molecular techniques to target methane-oxidizing bacteria in different environments
19 (Kolb et al. 2003; Pester et al. 2004), including landfill cover soil (Cebron et al. 2007).

20 Within the framework of a multidisciplinary study assessing the potential of a mix of sand and
21 compost as landfill cover soil, this study was designed to compare methanotroph dynamics,
22 diversity and potential activity between the existing silty cover soil and the substrate used in one
23 of 3 experimental PMOBs, herein referred to as PMOB-1. For this, several laboratory analyses

including methanotroph counts, diversity and potential CH₄ oxidation activity were undertaken. This comparison, based on biological parameters, provides a complementary approach to the physico-chemical characterization of the performance of the PMOB-1.

2. Materials and Methods

Experimental plot, materials and sampling: The PMOB-1 was constructed during the summer of 2006. The details and complete description of the instrumentation of this experimental plot can be found in Cabral et al. (2007) and Jugnia et al. (2008). Its dimensions are 2.75 m wide; 9.75 m long; 1.20 m deep. The following materials were employed: 0.8 m of substrate underlain by a 0.1 m thick transitional layer (6.4-mm net gravel) and a 0.2 m thick gas distribution layer (12.7 mm net gravel). The substrate layer consists of a mixture of sand and compost, composed of 5 volumes of compost (before sieving) and 1 volume of coarse sand ($D_{10} = 0.07$ mm; $D_{85} = 0.8$ mm; $C_u = 4.3$). After mixing, the material was sieved using a 12 mm industrial mesh. The particle size distribution of the substrate was: 0% of the particles with diameters between 5 and 12 mm, 29% of the particles with diameters between 2.5 and 5 mm, 15.7% between 1.25 and 2.5 mm, 14.6% between 0.63 and 1.25 mm, 34.9% between 0.08 and 0.63 mm, and 6.0% < 0.08 mm.

The reference soil (RS) is classified as silt and its particle size distribution was: 24.2% of the particles with diameters between 0.08 and 0.63 mm, and 75.8% < 0.08 mm.

The sampling method used PVC coring tubes (0.05 m diameter) to collect (monthly between June-December 2006 and May-December 2007) substrate and RS samples at three different locations and at different depths (0-0.1 m, 0.1-0.2 m, 0.2-0.3 m and 0.3-0.4 m). To prevent causing damage to the PMOB-1 structure, the holes were then filled with the same material (the

material used for construction of the PMOB-1) and compacted to avoid preferential gas paths. Also, the holes in the RS were filled with RS material (collected from the landfill) and compacted. The holes were marked to prevent reuse in the next sampling. Analyses including pH (determined in distilled water 1:3 v/v), gravimetric water content (w%) (CAN/BNQ 2501-170-M-86) and the organic matter content (OM) (ASTM D 2974 00) were determined in the laboratory.

Potential CH₄ oxidation rate: Experiments were performed in aerobic microcosms consisting of 10 g (wet weight) of soil in 125 ml glass serum bottles capped with Teflon-lined rubber stoppers and sealed with aluminum crimps. To each flask, 10 ml of pure CH₄ was added using a syringe. Samples were prepared in triplicate and incubated at room temperature (25°C) for 48h. The potential CH₄ oxidation rates were measured by collecting a time series (at 0 min, 30 min and every hour until the end of the test) of gas samples from the headspace of the serum bottles. CH₄ analyses were performed using a gas chromatograph (Agilent 3000A micro-GC) equipped with a thermal conductivity detector running at 45°C. For gas determination, 1 ml of the gas sample was injected into the GC system. A Poraplot-Q column (8 m x 0.32 mm) was used to determine the CO₂ concentration and a MS-5A molecular sieve (10 m x 0.32 mm) was used to determine the CH₄, O₂ and N₂ concentrations. Simultaneous integrations of peaks using the Cerity QA-QC software (Agilent) were acquired for gas concentrations.

Methanotroph counts: The most probable number (MPN) method was used for methanotroph counting. The test was carried out monthly between June 2006 and December 2007 for the PMOB substrate and from September 2006 to December 2007 for the reference soil. The most probable number of culturable methanotrophs in the sample was determined by serial tenfold dilutions in 96-well plates (microliter) containing a liquid mineral medium (Heyer et al.

2002; Heyer et al. 1984). The plates were incubated for 4 weeks at 30°C in gastight jars containing 3% CO₂ and 18% CH₄ in air.

DNA extraction and PCR amplification of *pmoA* genes: Total DNA was extracted using a laboratory protocol adapted from Fortin et al. (2004). To amplify methanotrophic DNA, characterized oligonucleotide primers were chosen to amplify a 500 bp conserved region of the particulate methane monooxygenase (pMMO) gene: A189f-GC and mb661r. The sequence of the GC-clamp was 5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G. The sequences of the primers were as follows: A189f: 5'-GGI GAC TGG GAC TTC TGG-3' and mb661r: 5'-CCG GIG CAA CGT CIT TAC C-3' (I = inosine). Reactions were carried out in a final volume of 50 µl, containing 10 µl of total DNA (undiluted, 1:10 or 1:50), 25 pmol of each oligonucleotide primer, 8 µl of 1.25 mM of dNTP, 5 µl of 25 mM MgCl₂ and 2.5 units of Taq polymerase (Invitrogen, Burlington, ON). The samples were denatured for 5 min, at 96°C, and then the Taq was added. A touchdown PCR was performed in which the annealing temperature was set at 60°C and decreased by 0.5°C, for every cycle, until it reached 50 °C. Denaturation was carried out at 94°C for 1 min followed by an annealing time of 1 min. Finally, primer extension was carried out at 72°C for 3 min and 14 additional cycles were conducted at 55°C. In total, 35 cycles were completed.

Denaturing gradient gel electrophoresis (DGGE) analysis: PCR products were concentrated by ethanol precipitation and 450 ng of DNA (*pmoA* gene) were applied to an 8% (wt/vol) acrylamide gel containing a 30 to 60% denaturant gradient. The 100% denaturant consisted of 7 M urea and 40% formamide. Gels were run at 60°C for 16 h at 80 V in 1 x Tris-Acetate-EDTA (TAE) buffer using a Bio-Rad Dcode universal mutation detection system. Gels

1 were stained for 30 min in Vistra Green (Amersham Biosciences Inc., Baie d'Urfe, QC) and
2 visualized with a FluorImager System model 595 (Molecular Dynamic, Sunnyvale, CA, USA).

3 **Phylogenetic tree:** Selected DGGE bands were excised from the gel and eluted in 60 µl of
4 MilliQ water at 4°C overnight. Fifty µl of the eluted DNA was purified with the QIAquick PCR
5 purification kit (QIAGEN Inc.). One µl of DNA was reamplified with the A189 and mb661
6 primers without the GC clamp as follows: an initial denaturation of 5 min at 96°C, followed by
7 25 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min. The PCR products were then
8 concentrated and purified with a QIAGEN kit. Sequencing of DNA samples was performed by
9 the « Laboratoire de synthèse et d'analyse d'acide nucleique » of Laval University (Ste-Foy, QC,
10 CA) with a capillary ABI Prism 3100 sequencer.

11 The *pmoA* gene sequences were aligned using MacVector 7.2 software and compared to the
12 available GenBank databases using the Fasta algorithm. The Phylogenetic tree was inferred by
13 the neighbour-joining algorithm and Jukes-Cantor method with gamma correction. The tree was
14 constructed using a MacVector 7.2 software package. The robustness of inferred topologies was
15 later tested by 1,000 bootstrap resamplings of the neighbour-joining data.

16 Sequences from this study have been deposited in the GenBank database under Accession
17 Numbers EU292151-EU292168 for substrate samples and EU679355-EU679361 for reference
18 soil samples.

19 20 3. Results and discussion

21 3.1. Substrate and reference soil properties

22 In 2006, the pH of the PMOB-1 substrate remained practically stable around an average value
23 of 7.2 ± 0.1 , at all depths (Fig. 1 a). In 2007 the pH values decreased progressively over time

1 stabilizing around an average value of 6.8 ± 0.3 ; the deep layers had slightly higher pH values
2 than the upper layers. Overall, the pH values obtained for the RS were a little higher than those
3 of the substrate. Moreover, the overall average of the pH values (7.6 ± 0.2) for the RS in 2007
4 was close to the only value (7.7 ± 0.07) recorded in 2006 (only one sampling date in September,
5 Fig. 1 b). From a spatiotemporal point of view, in contrast to the PMOB-1, the pH of the RS
6 seems to have varied very little over time but also as a function of sampling depth over the two
7 years of the study. In all cases, the PMOB-1 and the RS appeared to be favourable environments
8 for the development of methanotrophic bacteria; insofar as the optimum pH for the growth of
9 these microorganisms is between 5.5 and 8.5 (Dunfield et al. 2007; Hilger et al. 2000).

10 The decrease in the pH of the PMOB-1 substrate observed in 2007 may in part be related to
11 the CH_4 oxidation by the methanotrophic bacteria. A previous study by Hilger et al. (2000) in
12 laboratory columns, that had been gassed with landfill gas, showed that pH soil decrease from
13 6.3 at the bottom of the reactor to 5.2 at the top layer. Accumulation of intermediate products of
14 methane oxidation reactions and exopolymeric substances were the principal causes of the pH
15 decrease. This seems to be confirmed in this study, whereby the decrease in pH values was
16 generally greater in the upper layers, exactly where – due to the presence of O_2 – the potential
17 methane oxidation activity was higher (see discussion). In addition, it is known that the
18 decomposition of organic matter and the resulting production of carbon dioxide also contribute
19 to reducing the pH of soil (Bender and Conrad 1995; Hilger et al. 2000)

20 Over the two years of the study, the degree of water saturation values, S_r , of the PMOB-1
21 substrate varied between 57.5% and 100% (Mean \pm SD = $84.4\% \pm 9.7\%$), whereas for the RS the
22 degree of saturation varied between 44.6% and 83.3% (Mean \pm SD = $66.9\% \pm 10.2\%$). From a
23 spatiotemporal point of view, the S_r variations for the substrate were particularly large at the

1 surface (between 0 and 0.1 m; Fig. 2 a). Deeper in the profile, the Sr values remained not only
2 stable, but also very high. These high values may also be attributed to the water-retention
3 capacity of the organic matter rich substrate (compost) (He et al. 2008). For the reference soil in
4 2007, the Sr fluctuated a lot over time and for all depths (Fig. 2 b).

5 The organic matter (OM) content of the PMOB-1 substrate varied between 20.0% – 27.7%
6 (Mean \pm SD = 22.9% \pm 1.3%) and between 0.2% – 1.3% (Mean \pm SD = 0.9% \pm 0.3%) in the RS.
7 Low spatiotemporal variations of OM were observed in these two types of potential
8 methanotrophic bacterial supports (Fig. 3 a and b). Organic matter acts as an essential nutrient
9 reservoir for the development of methanotrophic bacteria (Czepiel et al. 1995). Also, the organic
10 matter is able to provide higher porosity for the cover (Humer et al. 2008). The difference of OM
11 content in the substrate and the RS will result in differences in methanotroph counts and
12 potential activity. Börjesson et al. (2004) found that using soil with a higher organic matter
13 content would result in increased methane oxidation. In this example (Börjesson et al. (2004), the
14 methane oxidation capacity of a mineral cover soil ($1.17 \mu\text{mol CH}_4 \text{ g.dw}^{-1}\text{h}^{-1}$) was considerably
15 lower than that from a previous study (Borjesson et al. (1998) using a landfill cover soil rich in
16 organic matter ($10.8 \mu\text{mol CH}_4 \text{ g.dw}^{-1}\text{h}^{-1}$).

18 3.2. Methanotroph counts

19 Over the two years of the study, the number of viable and culturable methanotrophic bacteria
20 in the PMOB-1 varied between 2.5×10^6 and 1.5×10^9 cells g dw⁻¹ (Mean \pm SD = $1.7 \pm 3.0 \times$
21 10^8 cells g dw⁻¹). These numbers are numerically higher by at least one order of magnitude, than
22 those obtained with the reference soil (range = 1.0×10^6 - 1.6×10^8 cells g dw⁻¹ and Mean \pm SD
23 = $5.2 \pm 4.9 \times 10^7$ cells g dw⁻¹). However, we did not notice any significant difference (t-test, $p >$

0.05) between the mean counts obtained for these two environments. The results of our counts with the PMOB-1 are comparable to the data of Gebert et al. (2003), which were 1.3×10^8 and 7.1×10^9 cells g dw⁻¹, obtained with a biofilter substrate. The number of methanotrophs in the RS was similar to the value obtained by Jones and Nedwell (1993) in landfill cover soils of 2.0×10^7 cells g dw⁻¹.

With the PMOB-1 substrate, we observed a decrease in the number of methanotrophs with sampling depth, the highest numbers generally being associated with the superficial layers between 0.0-0.1 m and 0.1-0.2 m (Fig. 4a). This was not always clear with the RS, both in 2006 and 2007 (Fig. 4b). According to Gebert et al. (2003), methanotrophs develop better in the upper layers where there is an optimum supply of oxygen. Therefore, it is likely that the difficulty of O₂ diffusing into the deepest layers, is one of the primary factors resulting in low methanotrophic bacterial development in these zones. Therefore, the more porous character of the PMOB-1 substrate compared to the RS would be one of the reasons explaining a more distinct gradient of methanotroph numbers as a function of depth within the PMOB-1. Moreover, the increased degree of saturation with depth, slightly more pronounced within the PMOB-1 (Fig. 2), would have limited the diffusion of O₂ because gas diffusion is 10⁴ times lower in water than in air (Yanful 1993).

From a temporal point of view, in 2006, the evolution over time of the number of methanotrophs in the RS did not fluctuate much compared to their development in the PMOB-1, which suggests 3 developmental phases for the different depths considered (Fig. 4a). During the first phase, which is an adaptation phase (between June and August), the numbers vary over time. Later, during the second phase (between August and November) or the actual growth phase, the number of bacteria significantly increased, especially at the surface (0.0 - 0.1 m), to

reach the maximum values recorded during 2006, somewhat resembling the profile observed with the RS during this time period. The third phase, in December, was characterized by a decline in the number of methanotrophs at all depths. In 2007, the number of methanotrophs in the PMOB-1 increased, except during the period between July and September when a significant decrease in counts occurred at practically all depths (Fig. 4a). The low concentration of CH₄ (< 5% at 0.1 m depth) recorded in August in the first 0.4 m of the PMOB-1 may explain the decrease in the number of methanotrophs. In September, the low water saturation (62%) of the substrate enhanced methane flow (CH₄ concentration was approximately 50% at 0.1 m), which in turn, prevented O₂ penetration into the PMOB-1. The low O₂ concentration (< 1% at 0.1 m) was likely a cause of the decrease in the methanotroph counts observed in September. Instead, the results obtained in 2007 with the RS indicated relatively stable methanotroph counts near the surface (0-0.1 m), which contrasts with the significant fluctuations over time observed at 0.1-0.2 m and 0.2-0.3 m and the constant increase in counts between June and November at 0.3-0.4 m. It is most likely that this decrease in temperature in December (< 5°C), in both years was responsible for the decline in the methanotroph population.

3.3. Methanotroph diversity

Molecular techniques - DGGE fingerprinting analyses and *pmoA* gene sequencing - were performed for a preliminary characterization of methanotroph communities present in samples from different depths in the PMOB-1 and RS. The DGGE analysis, illustrating changes in methanotroph diversity as a function of time and depths within the PMOB-1 and RS samples, is shown in Fig.5. On the DGGE gel, similar bands of PMOB-1 samples were identified by the same letters and by the same numbers on RS samples. DGGE patterns indicated a few dominant

bands for the substrate and RS, PMOB-V and RS-17 which melted at the higher denaturant concentrations and PMOB-D and RS-4 that denatured at the lower concentrations (in the upper part of the gels). The number of bands, on the PMOB-1 gel increased considerably over time near the surface (0.0-0.1 m) especially between October and December, while a slight increase in the number of bands was observed in November at the top layer of the RS. With the samples from 0.1-0.2 m and 0.3-0.4 m, an increase in the number of bands was less significant for the PMOB-1 than in the uppermost layer and did not seem to vary with time in the RS samples.

DGGE bands (*pmoA* gene) were sequenced and compared to the GenBank database. Potential *pmoA* sequence chimaeras were removed from the library analysis and only the proper sequences were used to construct the phylogenetic tree for both materials (Fig. 6). The scale bar in Fig. 6 indicates the bootstrap values (percentages) based on 1,000 replicates.

Dominant bands (PMOB-V and RS-17) excised from the PMOB-1 and RS gels were related to the genus *Methylocaldum*, a thermotolerant group of type Ib methanotrophs (*Gammaproteobacteria*) and were similar to uncultured *Methylocaldum* (99%; AY195659) found by Bodrossy et al. (2003) in landfill cover soil and to *Methylocaldum* sp. 5FB (96%; AJ868403) found by Knief et al. (2003) in mixed forest soil. Bands PMOB-D and RS-4 were related to the genus *Methylobacter*, a type Ia methanotroph (mesophilic bacterium) and were similar to uncultured bacterium (AJ868251) found by Knief et al. (2006) in German hydromorphic soil and to an uncultured bacterium (DQ059819) found by Lin et al. (2005) in Mono Lake (central California).

The DNA sequencing results from different gel bands revealed the presence of related or even identical species in the substrate and RS, which for the most part were related to the genus *Methylobacter*. However, other species related to type Ia methanotrophs were observed. We

noted species related to the genera *Methylomonas* (i.e. band PMOB-O), *Methylomicrobium* (i.e. bands PMOB-F/H and RS-8) and *Methylosarcina* (i.e. bands PMOB-J and R) (Fig. 6). Methanotroph diversity in landfill cover soil has been studied by many authors who reported the dominance of type I methanotrophs and the presence of the same genera (Bodrossy et al. 2003; Cebon et al. 2007; Wise et al. 1999). The inability to detect type II methanotrophs (genera *Methylosinus* and *Methylocystis*) in this study does not rule out their presence, since they may be below detection limits in our samples. Type II methanotrophs have been detected in other landfills using different molecular methods (Chen et al. 2007; Kallistova et al. 2007).

The molecular approach used to analyze methanotrophic bacteria in the PMOB-1 and RS samples does not provide quantitative data on their physiological activity. The data do indicate the presence of and relative diversity of methanotrophic bacteria in the total microbial community structure, and provide an indication of methane oxidation potential in the samples.

3.4. Potential CH₄ oxidation

The methane oxidation potential of the PMOB-1 ranged between 12 and 441 $\mu\text{g CH}_4 \text{ h}^{-1} \text{g dw}^{-1}$ (Mean \pm SD = $130 \pm 136 \mu\text{g CH}_4 \text{ h}^{-1} \text{g dw}^{-1}$) and varied with time and depth (Fig. 7a). This pattern of change in potential methane oxidation was also observed in the RS. However, the magnitude of variation in the RS was much less (Fig. 7b), ranging between 0 and 76 $\mu\text{g CH}_4 \text{ h}^{-1} \text{g dw}^{-1}$ (Mean \pm SD = $14 \pm 19 \mu\text{g CH}_4 \text{ h}^{-1} \text{g dw}^{-1}$), i.e. nearly five times lower than the range obtained for the PMOB. The values found in the present study are of the same order of magnitude as those obtained by Figueroa (1993), who studied a biowaste compost under laboratory conditions comparable to those of the present study, and obtained a maximum potential oxidation rate of 128 $\mu\text{g CH}_4 \text{ h}^{-1} \text{g dw}^{-1}$. In addition, the values obtained for the

PMOB-1 are also comparable to those obtained by He et al. (2008) for a “waste soil” from a simulated landfill reactor (180-216 $\mu\text{g CH}_4 \text{ h}^{-1}\text{g dw}^{-1}$). However, with the RS, the maximum value of our results is in the range of values reported by other authors for landfill cover samples with a texture comparable to that of the RS. For example, De Visscher (1999) obtained a potential activity of 26 $\mu\text{g CH}_4 \text{ h}^{-1}\text{g dw}^{-1}$ for a sandy loam cover soil; Scheutz and Kjeldsen (2004) obtained 118 $\mu\text{g CH}_4 \text{ h}^{-1}\text{g dw}^{-1}$ for a loam soil; and Börjesson and Svensson (1997) obtained 173 $\mu\text{g CH}_4 \text{ h}^{-1}\text{g dw}^{-1}$ for a silty loam soil.

From the physico-chemical point of view, although the organic matter content (which improved the porosity of soil) was low in the RS, this did not seem to interfere with the development of methanotrophs in the site cover. The methanotrophic bacterial counts (Fig. 4) were very similar between the RS and the PMOB-1. This demonstrates that the population density do not necessarily ensure a high CH_4 oxidation potential. In the RS, the low methanotroph activity is also attributable to the low amounts of molecular oxygen and methane, both essential to the methane oxidation reaction (He et al. 2008). During the sampling campaigns, it was impossible to collect gas samples from the probes installed beside the experimental plot, where the RS samples were collected. The gas probes were often in suction even at 0.1m deep. The occlusion of pores in the RS prevented the diffusion of gases (CH_4 and O_2) through the cover causing the poor activity of methanotrophic bacteria, which was the opposite of what was observed in PMOB-1.

The maximum rates of methane oxidation, for both PMOB-1 and the RS, were obtained in the uppermost layer (0.0 - 0.1 m) in July 2007, and were 441 $\mu\text{g CH}_4 \text{ h}^{-1}\text{g dw}^{-1}$ and 76 $\mu\text{g CH}_4 \text{ h}^{-1}\text{g dw}^{-1}$, respectively (Fig. 7a and b). Aside from this disparity between these maxima, the evolution over time of the potential methane oxidation of the PMOB-1 and the RS resulted in quite a rapid

1 decrease in values at all depths between June and August 2007, with the exception of the activity
2 recorded in July at the surface (0 - 0.1 m). This decrease might be explained by the low CH₄
3 concentrations between 0 and 0.4 m of the PMOB-1 during the period from 17 July to 13 August
4 2007. According to De Visscher et al. (1999), methanotrophic activity is influenced by the CH₄
5 load. Therefore, it is likely that the low concentration of substrate (CH₄) for the methanotrophs is
6 the main cause of the decreased activity measured at the beginning of August 2007.

7 From the month of August 2007, the CH₄ potential oxidation rate in the RS remained low (<
8 14 µg CH₄ h⁻¹g dw⁻¹) and practically constant over time and at all depths, which was quite
9 different from the activity observed during this period in the PMOB-1. In fact, between August
10 and December, the potential activity of the PMOB-1 remained relatively stratified, the highest
11 rates and greatest fluctuations taking place near the surface (0-0.1 m) compared to the deep
12 layers (0.1-0.4 m). However, all depths in the PMOB remained more active than the most active
13 layer of the RS during the same period. In September, there was a long dry spell, which had its
14 most pronounced effects on the uppermost layers of the substrate (Sr ≈ 62%; Table 1). The in
15 situ gas concentration measurements (from August 20th until September 6th) showed that the CH₄
16 concentrations at 0.1 m varied between 40 and 60%. However, during this same period, the O₂
17 concentration in the PMOB-1 was very low (< 1%), suggesting that the upward flow of biogas
18 was too high to allow proper aeration of the substrate. The lack of O₂ in September would have
19 caused methanotroph stress and resulted in the decrease in potential activity observed at the
20 surface (from 292 to 167 µg CH₄ h⁻¹g dw⁻¹) (Fig. 7a).

21 A slight increase in potential methane oxidation activity was observed in the uppermost layer
22 of PMOB-1 in October and November (158 and 256 µg CH₄ h⁻¹g dw⁻¹, respectively) (Fig. 7a).
23 This increase of potential activity was related to the increase in methanotroph counts (Fig. 4a).

1 Despite the high methanotroph counts in October and November, the potential methanotroph
2 activity was lower than that measured on July (which was the maximum potential activity). The
3 decrease in temperature observed in December ($< 5^{\circ}\text{C}$) seems to have influenced microbial
4 activity in the PMOB-1 substrate, since the CH_4 oxidation rates decreased abruptly, especially at
5 the surface where the values dropped from 256 to $56 \mu\text{g CH}_4 \text{ h}^{-1} \text{ g dw}^{-1}$. In other studies, it has
6 been shown that a decrease in the temperature results in a decrease in CH_4 consumption by
7 methanotrophs (Borjesson et al. 2004; Einola et al. 2007; Kettunen et al. 2006; Park et al. 2005).

8 The potential methanotroph activity studied in the first 0.4 m of PMOB-1 (Fig. 7a) showed the
9 presence of a CH_4 oxidation zone near the surface, particularly in the first 0.1 m of the substrate.
10 The absence of variability in the methane oxidation potential with depth in the RS (with the
11 exception of the values measured in July) (Fig. 7b), made it difficult to clearly show the
12 presence of an oxidation zone. The depth of the oxidation zone reported in the literature varies
13 depending on the authors. Gebert et al. (2003) and Urmann et al. (2007) reported, a CH_4
14 oxidation zone located in the first 0.15 m of a biofilter or laboratory columns, respectively. Other
15 authors have established that the oxidation zone may be deeper, at 0.3 m (De Visscher et al.
16 1999; Humer and Lechner 1999) and even at 0.5 - 0.6 m (Pawlowska and Stepniewski 2006).
17 The depth of the oxidation zone depends on the oxygen penetrating ability of the cover, which
18 itself is influenced by the degree of saturation of the soil. Near the surface of the PMOB-1, the
19 average degree of saturation was 76% (Table 1), while at depths below 0.2 m, the average value
20 increased to $>86\%$. At this latter degree of saturation, the air-filled pores are no longer
21 continuous (Nagaraj et al. 2006), rendering O_2 penetration very difficult. As a consequence, the
22 degree of saturation is a key factor explaining the localization of the observed oxidation zone in
23 the uppermost part of the PMOB-1.

4. Conclusions

The dynamics and changes in the community structure and the potential activity of methanotrophs, as a function of time and depth, in landfill cover materials were performed. This study took place in an experimental PMOB substrate (mix of compost and sand) and a reference soil which is an existing landfill cover soil (silty clay). The results demonstrated that the methanotrophic bacterial population densities were comparable in the PMOB-1 and the RS. Yet, the spatiotemporal evolution of their densities varied, in a different manner, for each of these two environments. The number of methanotrophs was practically stable in the RS, whereas a temporal evolution and stratification, with depth, was observed in the PMOB-1 substrate. The diversity of the methanotroph populations clearly showed an exclusive presence of Type I methanotrophs in the two environments under study. The potential methanotrophic activity trials indicated a greater activity in the PMOB-1 than in the RS, particularly close to the surface. This study reveals that the population density does not ensure a high CH₄ oxidation potential. The evolution, with time, of the potential methane oxidation of the PMOB-1 showed that methanotrophic potential activity was influenced by environmental factors (especially the degree of water saturation, CH₄ and O₂ concentrations).

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- 3

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1 FIGURES

2 Fig. 1

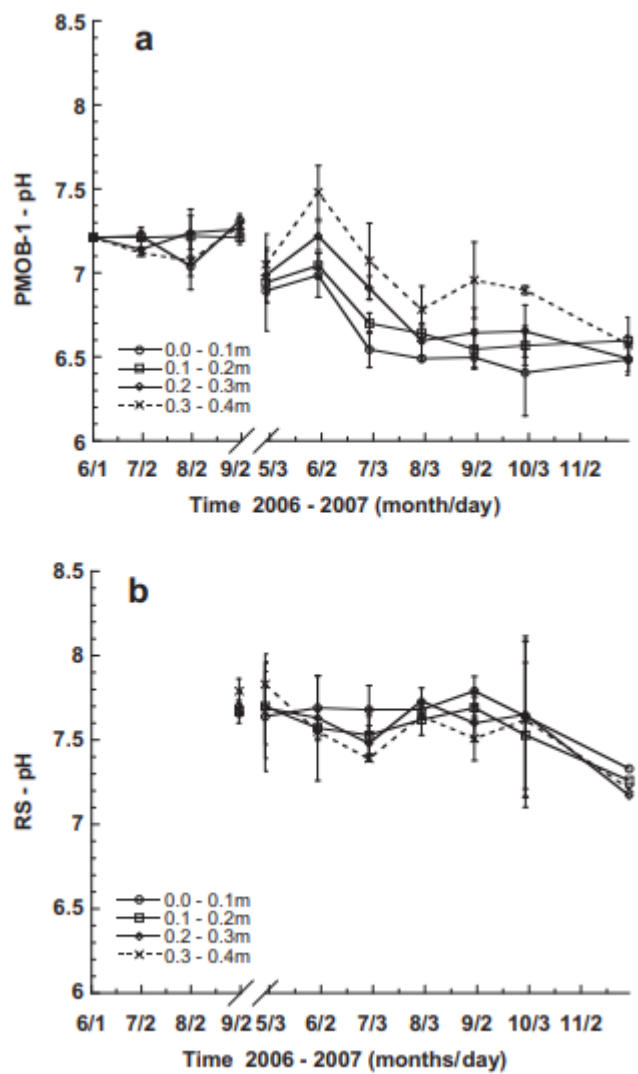


Fig. 1. Temporal pH evolution in the (a) PMOB-1 substrate and (b) existing cover soil (RS) at different depths. Data are averages of triplicates with error bars equivalent to 1 S.D.

1 Fig.2

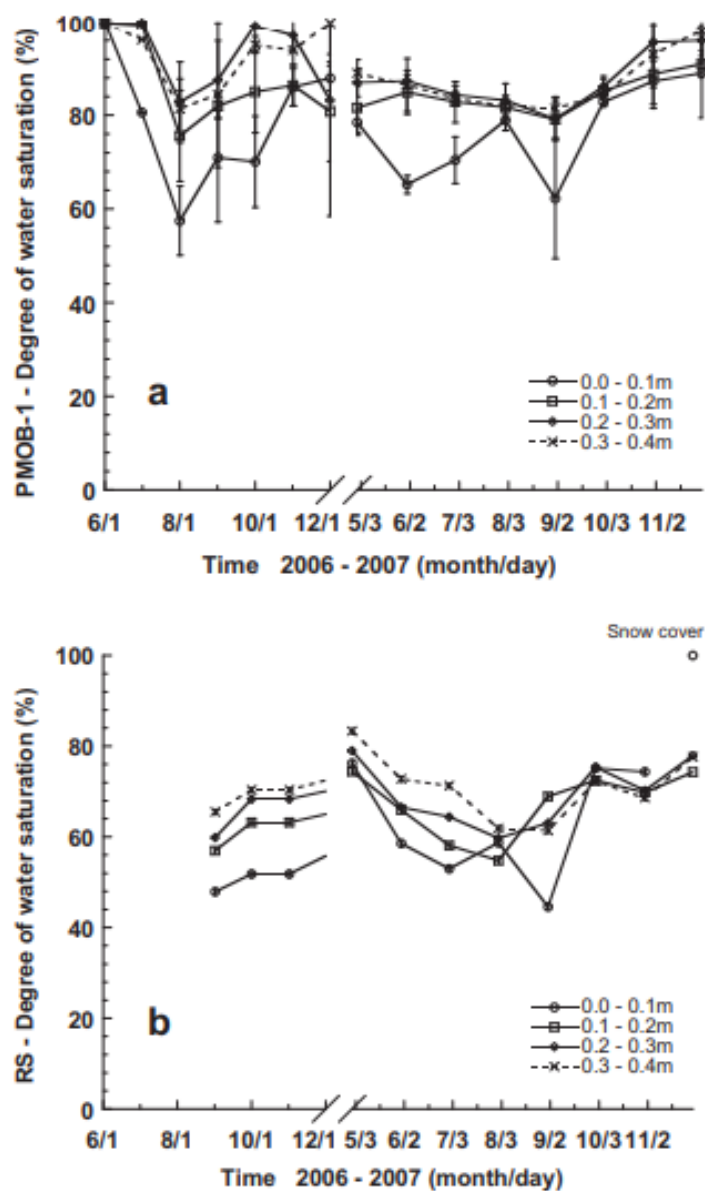


Fig. 2. Temporal degree of saturation evolution in the (a) PMOB-1 substrate and (b) existing cover soil (RS) at different depths. Data are averages of triplicates with error bars equivalent to 1 S.D.

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1 Fig.3

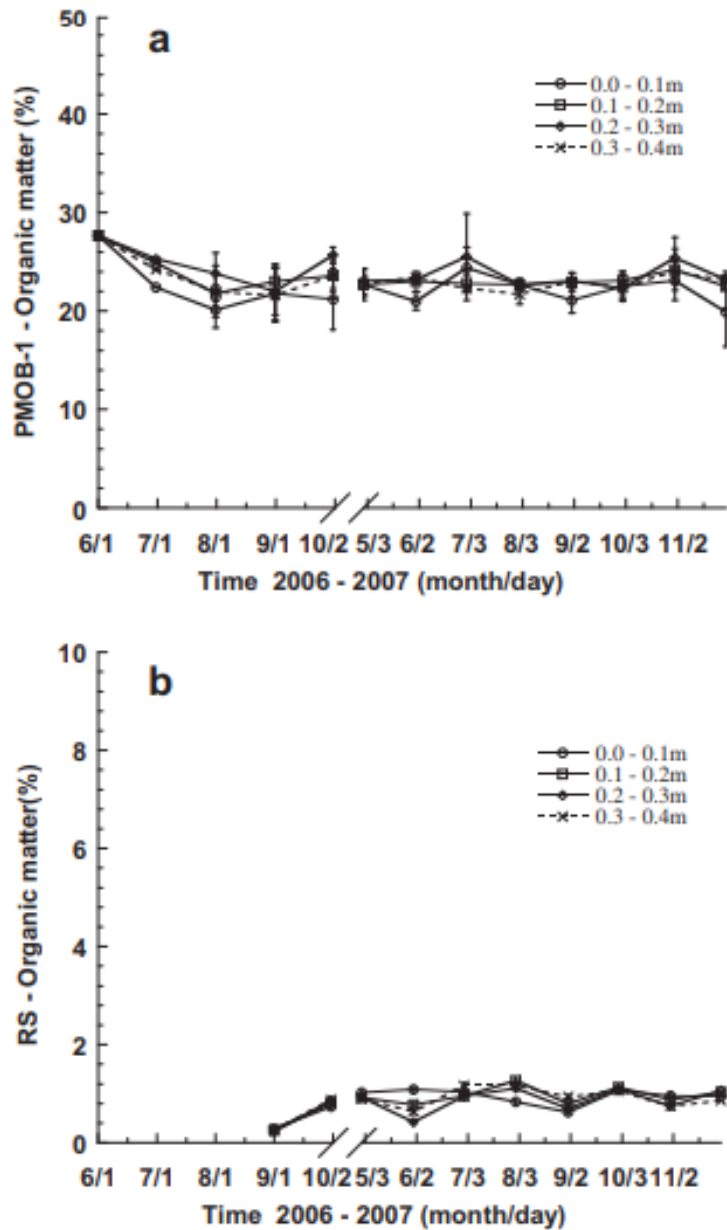


Fig. 3. Temporal organic matter content evolution in the (a) PMOB-1 substrate and (b) existing cover soil (RS) at different depths. Data are averages of triplicates with error bars equivalent to 1 S.D.

1 Fig.4

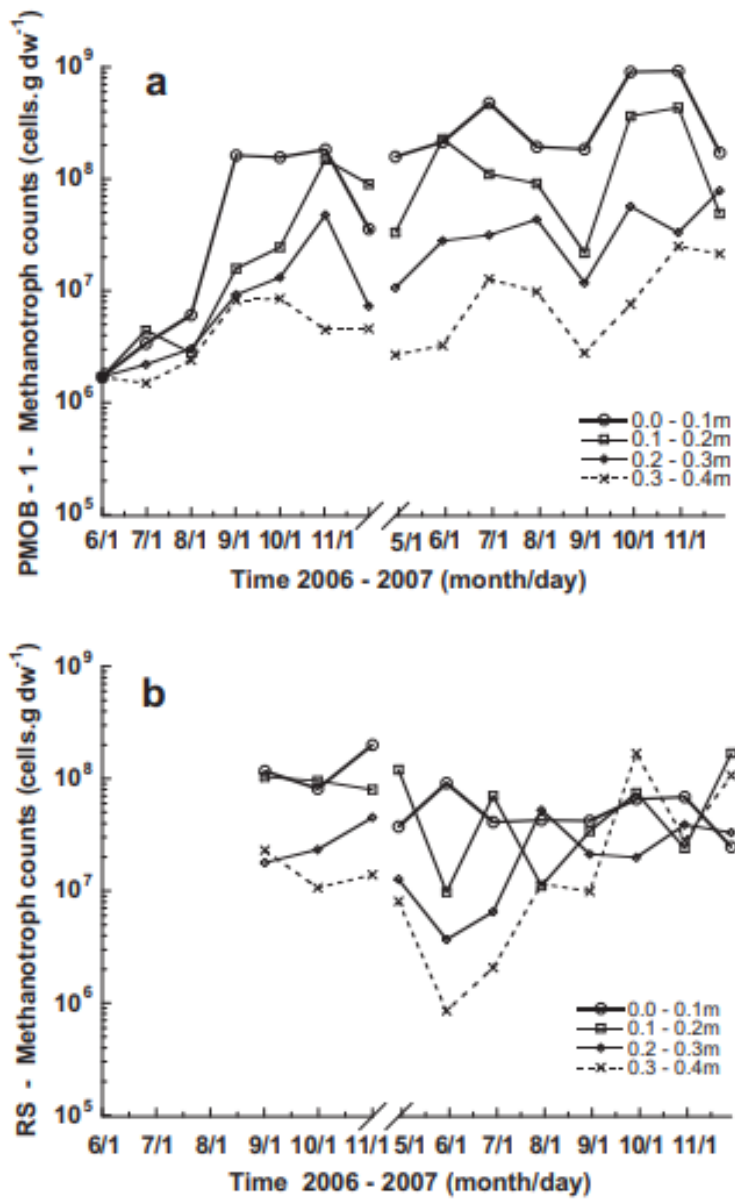


Fig. 4. Temporal methanotroph count evolution in (a) PMOB-1 substrate and (b) existing cover soil (RS) at different depths. Data are averages of triplicates with error bars equivalent to 1 S.D.

1 Fig.5

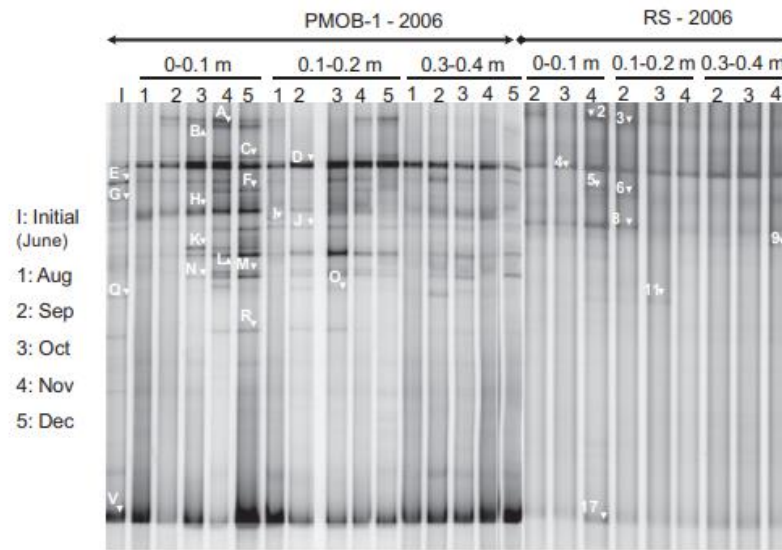
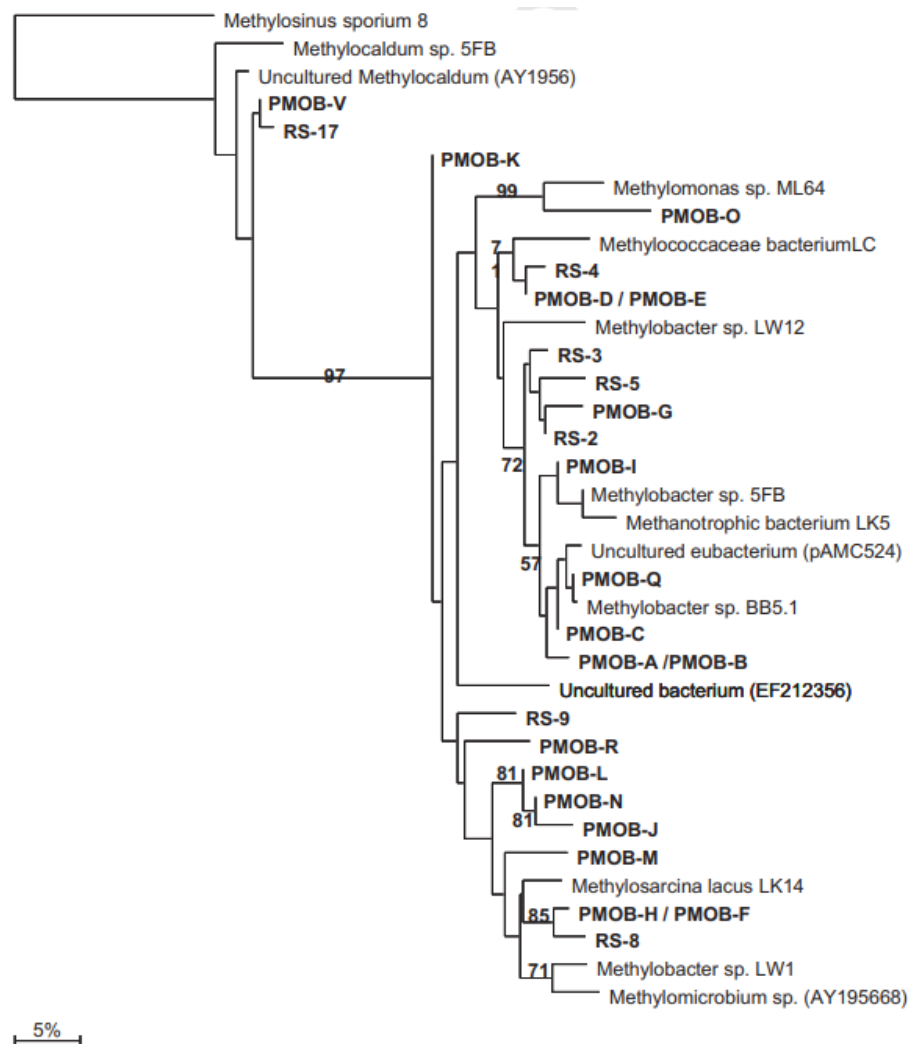


Fig. 5. *pmoA* DGGE profile from PMOB-1 and RS samples at different depths (0.0–0.1 m, 0.1–0.2 m and 0.3–0.4 m) between June and December 2006. The gel had a gradient of 30 to 60% denaturant. Lanes: I, initial material sampled in June; 1 in August; 2 in September; 3 in October; 4 in November and 5 in December.

1 Fig. 6



2 **Fig. 6.** Phylogenetic relationships of bacterial *pmoA* gene sequences of the PMOB-1 and RS DGGE bands. Numbers of the nodes are the bootstrap values (percentages) based
3 on 1000 replicates.

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1 Fig.7

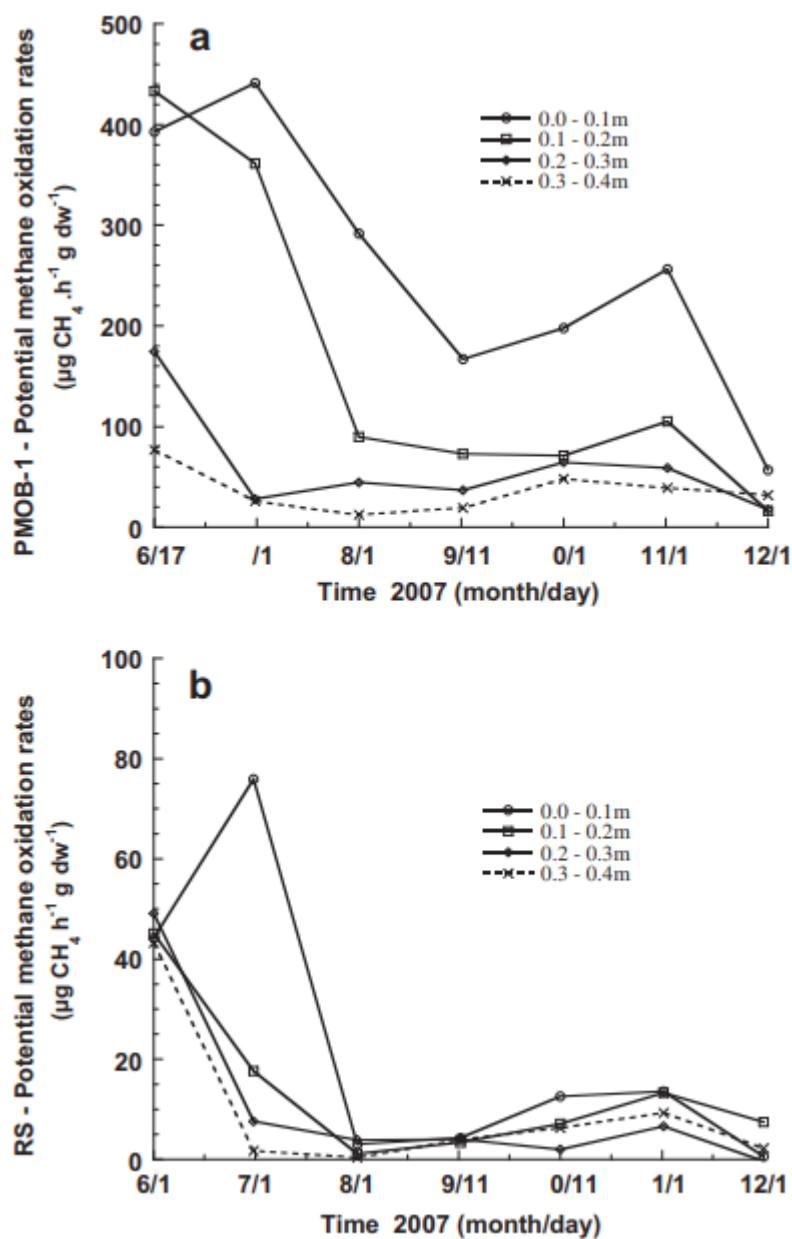


Fig. 7. Evolution of potential methanotrophic activity of (a) PMOB-1 substrate and (b) RS at different depths.

1 TABLE

Table 1

Summary of potential CH₄ oxidation (μg CH₄ h⁻¹ g dw⁻¹), degree of saturation (%) and soil temperature (°C). The values were observed between June and December 2007 in the uppermost layer (0.0–0.1 m) of PMOB-1.

Sampling dates	Potential CH ₄ oxidation (μg CH ₄ h ⁻¹ g dw ⁻¹)	Degree of saturation (%)	Soil temperature (°C) (daily average ± SD)
04-June-07	393.0	65.2	21.0 ± 0.3
09-July-07	441.1	70.4	25.7 ± 0.9
09-August-07	291.7	79.0	24.2 ± 0.8
09-September-07	167.0	62.3	29.8 ± 0.5
15-October-07	197.9	82.9	11.1 ± 0.2
08-November-07	256.3	87.3	6.7 ± 0.2
01-December-07	56.7	89.0	3.4 ± 0.0

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